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Defining Subsets of Naive and Memory B Cells Based on the Ability of Their Progeny to Somatically Mutate In Vitro

Debra J. Decker,* Phyllis-Jean Linton,*† Samantha Zaharevitz,* Matthew Biery,‡ Thomas R. Gingeras,‡§ and Norman R. Klinman*
*Department of Immunology
The Scripps Research Institute
La Jolla, California 92037
‡Life Sciences Research Laboratory
Baxter Diagnostics, Incorporated
4245 Sorrento Boulevard
San Diego, California 92121

Summary

The increased affinity of memory antibody responses is due largely to the generation and selection of memory B cells that accumulate somatic mutations after initial antigenic stimulation. Further affinity maturation and mutation also accompany subsequent immunizations. Previous studies have suggested that, like primary antibody-forming cell (AFC) clones, secondary AFC do not accumulate further mutations and, therefore, the origins of progressive affinity maturation remain controversial. Here, we report the generation of somatically mutated memory B cell clones in vitro. Our findings confirm the existence of a naive B cell subset whose progeny, rather than generating AFC, somatically mutate and respond to subsequent antigenic stimulation. Interestingly, upon stimulation, a subset of memory B cells also generates antigen-responsive cells that accumulate further somatic mutations.

Introduction

A major consequence of T cell–dependent antigenic stimulation is the generation of memory B cells whose antibody products exhibit higher affinity for antigen after both primary (Eisen and Siskind, 1964; Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987) and subsequent (Klinman et al., 1966; Berek and Milstein, 1987; Shlomchik et al., 1989) immunizations. This affinity maturation results, in part, from the selection of cells whose surface immunoglobulin (sIg) receptors exhibit increased affinity as a consequence of somatic hypermutation (Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987; Berek and Milstein, 1987; Shlomchik et al., 1989; Malipiero et al., 1987; Siekevitz et al., 1987; Bothwell, 1984), a process that occurs in specialized sites within the follicles of the spleen and lymph nodes, called germinal centers (GC) (Berek et al., 1991; Jacob and Kelsoe, 1992). The observation that not only primary immunization but also subsequent antigenic stimulations can produce responses of increasing affinity led to the assumption that memory B cells can continue to undergo somatic hypermutation and thereby generate successive generations of higher affinity memory cells (Berek and Milstein, 1987; Shlomchik et al., 1989). However, Siekevitz et al. (1987) refuted this assumption by showing that immunoglobulin genes of the progeny of memory B cells, while containing previously acquired mutations, do not continue to accumulate mutations during the course of antibody-forming cell (AFC) generation. Furthermore, several investigators have questioned the ability of memory B cells to generate GC and, consequently, subsequent generations of memory B cells (MacLennan and Gray, 1986; Gray, 1993).

To resolve this controversy and elucidate further the cellular basis of the production and propagation of memory B cells, we have analyzed AFC clones generated in limiting dilution fragment cultures (Linton et al., 1989, 1992). This culture system was coupled to RNA-specific nucleic acid amplification (Guatelli et al., 1990; Gingeras et al., 1990; Stillman et al., 1994) and polymerase chain reaction (PCR)-based sequencing methods sufficiently sensitive to assess somatic mutation in individual AFC clones. This experimental approach was used to evaluate the relative capacity of cells of various B cell subsets to generate memory B cells and undergo somatic hypermutation.

Splenic B cells of nonimmune mice have been subdivided by the criteria of their high or low expression of the cell surface heat-stable antigen (HSA) recognized by the J11d monoclonal antibody (Bruce et al., 1981; Linton et al., 1989, 1992; Yin and Vitetta, 1992; Wu and Ward, 1993). Upon antigenic stimulation, the major population of naive cells, which are HSA^{hi}, generates AFC but not memory cells, whereas cells isolated as HSA^{lo} are greatly enriched for precursors that generate memory B cells rather than AFC. Additionally, HSA^{lo} cells are unique among B cells of naive mice in their ability to originate GC (Linton et al., 1992).

For the current analysis, we have examined the monoclonal in vitro responses of the total B cell population of naive mice and HSA^{lo} B cells obtained from either naive or immunized mice. To facilitate the evaluation of somatic mutation, these studies analyzed the response to the haptenic determinant (4-hydroxy-3-nitrophenyl)acetyl (NP) of B cells from C.B20 mice (Igh^b), wherein much of the response involves λ light chain–bearing cells that utilize a restricted set of V_H genes (Makela and Karajalainen, 1977; Allen et al., 1987; Siekevitz et al., 1987; Bothwell, 1984; Jacob et al., 1991; Jacob and Kelsoe, 1992; Linton et al., 1989). The findings indicate that the majority of λ-bearing NP-responsive splenic B cells of both naive and immunized C.B20 mice generate AFC clones after a single course of antigenic stimulation and the heavy (H) chain variable (V) regions of the AFC progeny of these cells did not accumulate somatic mutations in vitro. However, the responses of HSA^{lo} precursors obtained from naive mice,

†Present address: San Diego Regional Cancer Center, 3099 Science Park Road, San Diego, California 92121
§Present address: Affymetrix, 3380, Central Expressway, Santa Clara, California 95051

Fx[a]	VH	D	J	V/D/J junction[b]	bases/ colony	# mut. shared	*in vitro* mut/10^3 bp	# E.coli colonies	observed mutations
2	186.2	DQ52	4	TGT GCA AGA TC/G GCT AAC TGG G/AT GCT ATG GAC TAC TGG	198	0	0.4	11 1	none 91(TAT→TAC)
8	186.2	DFL16.1	4	TGT GCA AGA T/AC TAC GGT ACC G/AT GCT ATG GAC TAC TGG	200	0	0	7	none
9	186.2	DST4	4	TGT GCA AGA TC/A GGC TTA GCT CGG GGC ATT GAC CTT T/TG GAC TAC TGG	207	0	0	4	none
10a	186.2	?	4	TGT GCA AGA /CG/T GCT ATG GAC TAC TGG	186	0	0	4	none
10b	C1H4	DFL16.1	4	TGT GCA AG/T ACT ACG GGC TT/A TAT TAC TAT GCT ATG GAC TAC TGG	204	0	0.5	8 1	none 73(AAA→AAG)
11a	165.1	DSP2.2	2	TGT GCA /GAT TGG GAG ATT ACG CCA AGG ACT ATT /TTT GAC TAC TGG	204	0	0	8	none
11b	186.2	DFL16.1	1	TGT GCA AG/C CGA GGT TAC TAC GGT AGT AAG /TGG TAC TTC GAT GTC TGG	207	0	0	8	none
33[c]	186.2	DSP2.5,7,8	2	TGT GCA AGA /CAA ACT TTC /GAC TAC TGG	186	0	0	14	none
				AVERAGES		0.0	0.12		

Figure 1. H Chain V Region Sequences of AFC Clones Generated after One Course of In Vitro Antigenic Stimulation of Naive Splenic B Cells

(a) All fragment cultures had been stimulated for the first 3 days of culture (days 0–3) and produced λ-bearing IgG anti-NP antibodies by day 8 of culture. All fragments were harvested on day 13 of culture, except fragment 33, which was harvested on day 10.

(b) The data presented include the sequence from codon 92 of the V_H through codon 103 of the J_H (according to Kabat et al., 1991). Underlined nucleotides represent possible P nucleotides (LaFaille et al., 1989). Also presented are the total number of bases sequenced for each clone, the number of E. coli colonies from which a given germline or mutated sequence were obtained, the number of shared mutations, and the total number of in vitro generated mutations normalized to 10^3 sequenced nucleotides for each clone.

(c) The sequence presented for fragment 33 was obtained from two separately amplified aliquots of extracted RNA.

as well as a subset of the responses of B cells obtained from immunized mice, required two courses of antigenic stimulation before generating AFC clones and, in both cases, the H chain V region sequences of the resultant AFC clones included somatic mutations that had been generated in vitro.

Results

The Progeny of Primary AFC Precursors Do Not Accumulate Somatic Mutations

Previous findings from numerous laboratories have demonstrated that the V region genes of naive AFC precursors are not somatically mutated and that these genes do not accumulate somatic mutations in the AFC progeny of these cells. To establish a baseline for further studies of in vitro generated somatic mutations, we first analyzed the H chain V region sequences of AFC clones generated in limiting dilution fragment cultures of B cells obtained from the spleens of naive C.B20 (Igh^b) mice after a single in vitro stimulation with NP_{10}–Limulus polyphemus hemocyanin (Hy). Consistent with results of earlier studies (Linton et al., 1989), 0.9 clones producing λ-bearing anti-NP antibodies were detected per 10^6 injected B cells within 8–10 days after stimulation. To ensure comparable analyses of sequences derived from naive AFC precursors with those of other precursor cell subsets, fragments were not harvested for sequence analysis until day 13 of culture. Additionally, although isotype switching is not required for somatic mutation (Shlomchik et al., 1989; Linton et al., 1989), any skewing of results associated with differences in immunoglobulin isotype was eliminated by limiting all analyses of both primary and memory AFC clones to those producing immunoglobulin G (IgG) antibodies. Furthermore, oligonucleotide primers that annealed to the γ constant region were used for amplification.

As seen in Figure 1, 6 of 8 analyzed AFC clones utilized rearrangements of the V_H186.2 gene segment, which normally predominate among λ-bearing anti-NP antibodies of Igh^b mice (Allen et al., 1987; Siekevitz et al., 1987; Bothwell, 1984; Jacob et al., 1991). The remaining two clones utilized closely related members of the J558 V_H gene segment family. All members of a responding AFC clone share identical V_H–D–J_H junctions, while members of separate AFC clones differ in this third complementarity determining region (HCDR3). By these criteria, 4 of 6 analyzed fragments contained a single responding AFC clone, while the remaining two fragments contained two distinct AFC clones. Only two potential point mutations among clone members were observed among the 66 sequences obtained. This number of mutations is fewer than anticipated from amplification errors alone (see Experimental Procedures). Thus, among the nucleic acid sequences obtained from 8 clones of primary AFC generated in vitro, there was neither significant variation from the sequences of the rearranged germline V gene segments nor significant variation among the multiple sequences obtained from any of the resultant AFC clones. These findings confirm earlier conclusions concerning the absence of somatic mutations in V regions of primary AFC precursors and their AFC progeny (Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987), and establishes the fidelity of the amplification procedures used to analyze the H chain V region sequences of in vitro generated AFC clones.

The Progeny of Naive HSA^{lo} Memory Progenitors Accumulate Somatic Mutations In Vitro

Somatic mutations have been shown to accumulate during the generation of memory B cells (Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987; Berek and Milstein, 1987; Shlomchik et al., 1989; Malipiero et al., 1987; Siekevitz et al., 1987). To establish the competence of splenic

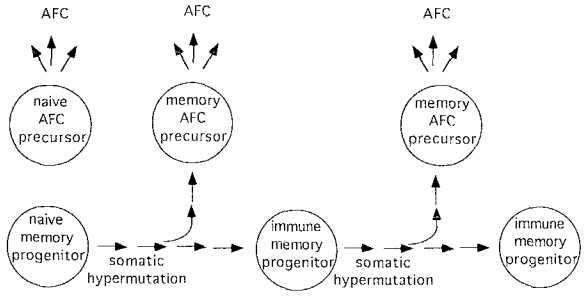

Figure 2. Proposed Pathway for the Generation and Propagation of Memory B Cells

Two functionally distinct B cell subsets are found in the spleen of naive mice: naive AFC precursors, which, upon antigenic stimulation, generate AFC clones in the absence of somatic hypermutation; and naive memory progenitors, which are enriched among cells expressing low levels of HSA, and which generate somatically mutated memory B cells rather than AFC after a single course of T_H-dependent antigenic stimulation. Memory B cells that are both somatically mutated and isotype switched can also display two distinct functional phenotypes in that, while the majority respond to a single course of T_H-dependent antigenic stimulation by the generation of an AFC clone without the further accumulation of somatic mutations, other memory B cells do not generate AFC after such stimulation but, instead, generate higher order memory B cells that accumulate additional somatic mutations. We hypothesize that the two distinct types of memory B cell responses are the consequence of the existence of two distinct precursor cell subsets, one of which can generate only AFC (memory AFC precursor), while the other (immune memory progenitor) generates progeny that somatically hypermutate and respond to subsequent antigen challenge. By this hypothesis, the latter population in immunized mice would be responsible for the propagation of memory responses.

fragment cultures to support somatic mutation and the capacity of HSA^{lo} precursors to generate memory B cells with somatically mutated V region genes, we analyzed the H chain V region sequences of AFC clones generated from naive HSA^{lo} splenic B cells after stimulation (days 0–2) and restimulation (days 6–8) in fragment cultures. As described previously, the vast majority of fragment cultures reconstituted with naive HSA^{lo} precursors yielded no detectable antibody after the first course of antigen stimulation (Linton et al., 1989, 1992; Wu and Ward, 1993). However, 4–6 days after the second course of in vitro stimulation, λ-bearing AFC clones were detected at a frequency of $0.6/10^6$ injected B cells. The requisite for two courses of antigenic stimulation to obtain AFC has been interpreted as a need for an initial stimulation to enable the generation of memory cells and a second stimulation to drive these cells to AFC formation (Linton et al., 1989; see Figure 2).

H chain V region sequences were obtained from the RNA of six distinct AFC clones (as determined by unique V_H–D–J_H junctions) generated from four fragment cultures reconstituted with naive HSA^{lo} precursors that underwent two courses of in vitro antigen stimulation (Figure 3). The set of sequences derived from each AFC clone shared a V_H–D–J_H junction but differed from one another by one to several nucleotides. Importantly, for each of the clones, at least one sequence was obtained that represented a rearrangement of unmutated H chain V gene segments. These unmutated H chain V gene rearrangements presumably represented the H chain V regions expressed by the naive HSA^{lo} precursor cells that originated the fragment culture clones. That the naive precursors of memory B cells are unmutated is consistent with sequences obtained from pools of HSA^{lo} precursors (D. J. D. and N. R. K., unpublished data) and with conclusions drawn by numerous investigators that somatic mutation is an antigen-driven process (Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987; Berek and Milstein, 1987; Shlomchik et al., 1989). In addition to unmutated sequences, each clone contained several sequences that differed from the germline sequence by at least 1 and as many as 5 nt. This finding indicates that multiple cells with unique mutations arose from a single progenitor after stimulation in fragment culture. Indeed, in 4 of 6 clones precursor progeny relationships could be established by the patterns of sharing of one or more mutated nucleotides. On the average, the sequences derived from these clones had 5.4 mutations/10^3 nucleotides, which is significantly higher than the number of nucleotide differences anticipated for errors due to the amplification procedures ($p < 0.01$, student's t test of multiple planned comparisons). These findings demonstrate the competence of the fragment culture system to enable somatic hypermutation and confirms the capacity of HSA^{lo} precursor cells to generate somatically mutated memory B cells.

Memory B Cells Display Two Distinct Response Phenotypes

To determine whether secondary B cells can propagate further generations of higher order (tertiary, quaternary, etcetera) memory B cells and, in the process, continue to accumulate somatic mutations, spleen cells were obtained from C.B20 mice that had been immunized with NP–Hy 1–2 months previously. To minimize contamination with naive AFC precursors that might have coexisted with memory B cells in these spleens, HSA^{lo} B cells were isolated for these analyses as memory B cells are enriched in the HSA^{lo} B cell population of immunized mice (Bruce et al., 1981). Additionally, in some instances, HSA^{lo} cells were also harvested on the basis of low to negative expression of sIgD, sIgM, or both. Following in vitro stimulation of HSA^{lo} B cells obtained from immunized mice, the responses observed fell into two distinct categories. A majority of the λ-bearing AFC responses (10 per 10^6 injected HSA^{lo} memory B cells) were characterized by antibody formation within 7 days of the initial (days 0–2) stimulation. However, an additional 6 responses per 10^6 injected HSA^{lo} memory B cells could be identified that produced no antibody after the initial stimulation but did produce λ-bearing antibody within 4–6 days after a second (days 8–10) in vitro stimulation. Thus, 37% of responses of memory B cells required two courses of antigenic stimulation to generate AFC, and the frequency of such responses was 10-fold higher than that for HSA^{lo} cells of naive mice.

Figure 4 presents the sequences obtained from the RNA extracted from fragment cultures of memory B cells (AFC precursors) that produced antibody after a single course of antigenic stimulation. All of these AFC clones contained

Fig[a]	VH	D	J	V/D/J junction[b]	bases/ colony	# mut. shared	*in vitro* mut/10^3 bp	# E.coli colonies	observed mutations
4a	186.2	DFL16.1	4	TGT GCA AGA /AGA GGG AGT ACT ACG GTA GTA GCC CAA /TAT GCT ATG GAC GAC TGG	213	0	4.0	3	none
								1	J(GAC→GGC)
								2	62(AAG→AAT)
								1	61(GAG→GAA),62(AAG→AAT),65(AGC→GGC)
4b	4M104	DFL16.2	4	TGT GCC TTT TAT TAC GGC TAC GGG /ATG GAC TAC TGG	195	0	9.2	1	none
								2	80(ATG→ATC)
								1	53(TAC→TCC),60(AAT→CAT),68(ATA→AGA)
								1	53(TAC→TCC),60(AAT→CAT),68(ATA→AGA), 69(TTG→CTG)
5	24.8	DSP2.?	4	TGT GCA AGA /CAC TAC TAT GCT AAC /TAC TAT GCT ATG GAC TAC TGG	204	0	6.5	2	none
								1	65(GGC→AGC)
								1	65(GGC→AGC),69(TTG→GTG),93(GCA→TCA)
								2	93(GCA→TCA),D(GCT→GCC)
6a	186.2	DSP2.2	4	TGT GCA AGA T/TC CCT AAT GAT TAC GAC GGG /ATG GAC TAC TGG	201	0	4.2	2	none
								2	64(AAG→GAG)
								1	81(CAG→CAT)
								1	81(CAG→GAT)
6b	V23	DFL16.1	1	TGT GCA AG/G AAA TTA CTA CGG TAC CC/C TTC GAT GTC TGG	198	0	2.2	4	none
								1	82c(CTG→CGG)
								2	61(GAG→AAG)
7	165.1	?	1	TGT GCA AGA T/YA CT/C TAC TTC GAT GTC TGG	195	0	6.2	2	none
								1	83(ACA→GCA)
								1	65(AGC→GGC),71(GTA→CTA)
								1	58(AAC→AAA),71(GTA→CTA),91(TAC→TAT)
				AVERAGES		0	5.4		

Figure 3. H Chain V Region Sequences of AFC Clones Generated after Two Courses of In Vitro Antigenic Stimulation of Naive HSA^{lo} Memory Progenitors

(a) All fragments were stimulated twice in culture (days 0–2 and days 6–8) and produced λ-bearing IgG anti-NP antibodies 4–6 days after the second course of stimulation. All fragments were harvested between days 13 and 16 of culture.
(b) As for Figure 1.

at least five base differences from the germline sequences of the rearranged germline gene segments. However, all of the sequences obtained from each clone were completely homologous. Therefore, the clonal progeny of each memory precursor that gave rise to AFC after a single in vitro stimulation shared a set of V region somatic mutations, which presumably represented the mutations accumulated during the in vivo generation of the precursors that originated the AFC clones. However, no further mutations were accumulated during the course of AFC clone generation in vitro.

Figure 5 presents the H chain V region sequences obtained from the AFC clones generated from the subset of memory B cells whose progeny produced detectable antibody only after two courses of in vitro stimulation. As with the sequences derived from the progeny of memory B cells that produced AFC after a single in vitro stimulation, all sequences derived from a given clone (as defined by an unique V_H–D–J_H junction) shared a set of nucleotides that differed from the germline of the rearranged germline gene segments. Again, these shared mutations presumably represented those obtained during the in vivo generation of the memory precursor that originated the clones. However, superimposed on these shared mutations was a set of additional mutations that differed among the sequences obtained from the cells comprising each individ-

Fig[a]	VH	D	J	V/D/J junction[b]	bases/ colony	# mut. shared	*in vitro* mut/10^3 bp	# E.coli. colonies	observed mutations
13	186.2	DFL16.1	2	TGT GCA AGA T/AC TCA TAT TAC TAC GGT AGT AGT TT/C TTT GAC TAC TGG	207	4	0	9	[52a(CCT→CCA),61(GAG→GCG),62(AAG→AAT), 82a(AGC→AAC)]
23	186.2	DFL16.1	3	TGT GCA /GGC TAT TAT TAC GGT CGT AGC CTC /TTT GCT TAC TGG	201	7	0	7	[62(AAG→AAA),64(AAG→AGA),65(AGC→AAC), 73(AAA→AAG),D(TAC→TAT),D(AGT→CGT)]
30	4M5	DSP2.5,7,8	4	TGT GCA AGA T/TT ATC TTA AGG TAA CTT CGA CTA T/CT ATG GAC TAC TGG	207	5	0	7	[52(AAT→TAT),53(AGC→ACC),62(AAG→AAA), 65(AGC→ACC),66(AAG→AGG),71(GTA→GTG), 73(AAA→GAA)]
31	593.3	DSP2.9	3	TGT GCA AG/C GTA GAT GGT CAC TAC GGT /TGG TTT GCT TAC TGG	201	8	0	5	[50(GAG→GTG),54(GAT→GAA),55(AGC→AAT), 57(ACT→ATT),58(AAC→AAG),73(AAA→AGA), D(GAC→CAC)]
34[d]	24.8	DFL16.1	2	TGT GCA AGA T/AT TAC TAC GGT AGA AG/C TAC TTT GAC TAC TGG	186	5	0	8	[64(AAG→GAT),71(GTA→ATA),74(CCC→TCC), D(AGT→AGA)]
40[c]	186.2	DFL16.1	4	TGT GCA AGA T/AT TAT TAC TAC TAT TT/T ATG GAC TAC TGG	198	1	0	28	[58(AAG→ATA)]
47[c]	186.2	DFL16.1	2	TGT GCA AGA T/AT GAT TAC TAC GGT GGT ACC TCC AC/C TTT GAC TAC TGG	207	4	0	16	[65(AGC→ACC),73(AAA→AAT,85(GAG→GAA), J(TAC→TCC)]
				AVERAGES		4.9	0		

Figure 4. H Chain V Region Sequences of AFC Clones Generated after One Course of In Vitro Antigenic Stimulation of HSA^{lo} Memory B Cells

(a) All fragments were stimulated for the first 2 days of culture (days 0–2) and produced λ-bearing IgG anti-NP antibodies by day 8 of culture. All fragments were harvested on day 8 of culture.
(b) As for Figure 1.
(c) The sequences presented for fragments 40 and 47 were each obtained from two separately amplified aliquots of extracted RNA.
(d) Fragments 13 and 34 were derived from a mouse reconstituted with HSA^{lo} memory B cells that were surface κ negative and fragment 23 was derived from a mouse reconstituted with HSA^{lo} memory B cells that were IgD^{lo}.

Fig[a]	VH	D	J	V/D/J junction[b]	bases/ colony	# mut. shared	*in vitro* mut/10^3 bp	# E.coli. colonies	observed mutations
16[c,d]	186.2	DST4	2	TGT GCA AGA /GGA CAG CTC AGA CTA C/AC TTT GAC TAC TGG	198	2	5.7	2 4† 6† 1 3†	[58(AAG→AGA)] [58(AAG→AGA)],57(ACT→GCT) [58(AAG→AGA)],N(GGA→CGA) [58(AAG→AGA)],N(GGA→CGA),74(CCC→TCC) [58(AAG→AGA)],N(GGA→CGA),59(TAC→GAC)
17[c,d]	4M114	DSP2.2	4	TGT ACA CGA T/TG GGC TAC TAT GAT TAC TCT /TAT CTT ATG GAC TAC TGG	207	5	8.0	5† 8† 3† 4† 1 1 1	[45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)] [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], J(GCT→GTT) [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], J(GCT→GTT),D(TAC→TGC) [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], J(GCT→GTT),D(TAC→TGC),52a(CCT→CTT) [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], J(GCT→GTT),D(TAC→TGC),52a(CCT→CTT),74(CCC→GCC) [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], 55(AGT→GGT),69(TTG→TTC),70(ACT→GAT) [45(CTT→CTG),62(AAG→AAC),80(GTG→ATG),82(CTC→TTC),94(AGA→CGA)], 64(AAG→ACG),88(GCG→ACA),89(GTC→ATC)
20[d]	4M108	DSP2.8	4	TGT ACA AGA /GA/G GAC TAC TGG	180	4	22.2	1 1 3 2	[50(GAG→AAG),53(AGC→CCC),76(AGC→ACC)] [50(GAG→AAG),53(AGC→CCC),76(AGC→ACC)],56(GGT→GCT),58(AAC→ACC) [50(GAG→AAG),53(AGC→CCC),76(AGC→ACC)],56(GGT→AAT),57(ACT→GCT), 59(TAC→TTC) [50(GAG→AAG),53(AGC→CCC),76(AGC→ACC)],56(GGT→AAG),57(ACT→TCT), 59(TAC→TTC),60(AAT→AAG),61(GAG→CAG)
21	186.2	DSP2.10	2	TGT GCA AGA /AGG GGA GTC TAC TAT AGT AAC C/AC TTT GAC TAC TGG	204	2	1.2	3 1	[58(AAG→AGG),63(TTC→TTA)] [58(AAG→AGG),63(TTC→TTA)],91(TAT→TAC)
22	186.2	DFL16.1	2	TGT GCA AGA T/AT TAC TAC GGT CGT AGT TC/C TTT GAC TAC TGG	201	1	0.8	5 1	[D(AGT→CGT)] [D(AGT→CGT)],73(AAA→GAA)
24	186.2	DFL16.1	2	TGT GCA AGA T/AC GAT TAC TAC GGT AGT AGG TAC TAC TTT CAC TAC TGG	207	2	0	9	[57(ACT→ACC),J(GAC→CAC)]
25[d]	186.2	DFL16.1	2	TGT GCA AGA T/AT TAC TAC GGT AGA AGC TAC TTT GAC TAC TGG	201	2	7.5	1 1 6 1 3	[83(ACA→GCA),D(AGT→AGA)] [83(ACA→GCA),D(AGT→AGA)],80(ATG→AGG) [83(ACA→GCA),D(AGT→AGA)],66(AAG→CAG) [83(ACA→GCA),D(AGT→AGA)],66(AAG→CAG),69(CTG→CAG) [83(ACA→GCA),D(AGT→AGA)],53(AAT→AGT),65(AGC→ACC),66(AAG→AGG)
26[d]	186.2	DFL16.1	2	TGT GCA AGA TC/C CAT TAC T/TC GGT AGT GGG TCC C/AC TTT GAC TAC TGG	207	10	3.6	1 3	[50(AGG→AGA),53(AAT→TAT),58(AAG→AGG),65(AGC→ACC),71(GTA→GTG), 76(AGC→AAC),78(GCC→ACC),80(ATG→ATA),D(TAC→TTC),J(GAC→AAC)] [50(AGG→AGA),53(AAT→TAT),58(AAG→AGG),65(AGC→ACC),71(GTA→GTG), 76(AGC→AAC),78(GCC→ACC),80(ATG→ATA),D(TAC→TTC),J(GAC→AAC)], 94(AGA→GGA)
27a[c,d]	186.2	?	1	TGT GCA AGA /AGA GGG AAT CCG C/AC TGG TAC TTC GAT GTC TGG	201	3	0	29†	[57(ACT→ATT),62(AAG→AAA),76(AGC→AAC)]
27b[c,d]	186.2	DFL16.1	2	TGT GCA AG/G TAT TAC TAC GGT GGT AGT TTC /GAC TAC TGG	198	5	1.4	8 1 1	[65(AGC→AGT),76(AGC→AAC),85(GAG→GAA),D(AGT→GGT),J(TAC→TAT)] [65(AGC→AGT),76(AGC→AAC),85(GAG→GAA),D(AGT→GGT),J(TAC→TAT)], 86(GGT→GGG) [65(AGC→AGT),76(AGC→AAC),85(GAG→GAA),D(AGT→GGT),J(TAC→TAT)], 66(AAG→ATG)
				AVERAGES		3.6	5.0*		

Figure 5. H Chain V Region Sequences of AFC Clones Generated Only after Two Courses of In Vitro Antigenic Stimulation of HSA^{lo} Memory B Cells

(a) All fragments were stimulated twice in culture (days 0–2 and 8–10) and produced λ-bearing IgG anti-NP antibodies 4–6 days after the second course of stimulation. All fragments were harvested between days 14 and 16 of culture.

(b) As for Figure 1.

(c) The RNA extracted from fragments 16, 17, and 27 was separated into two aliquots prior to amplification and the sequences designated with daggers were obtained from both aliquots.

(d) Fragments 16, 17, and 20 were derived from a mouse reconstituted with HSA^{lo} memory B cells that were surface κ negative. Fragments 26, 25, and 27 were derived from mice reconstitutes with HSA^{lo} memory B cells that were sIgD^{lo}, sIgD^{lo} and sIgM^{-}, and IgM^{-} respectively.

ual AFC clone. As with AFC clones derived from HSA^{lo} naive precursors, in several of the clones a precursor–progeny relationship could be established by the pattern of sharing of nucleotide differences. To verify that both the set of shared mutations and the unique mutations were present prior to nucleic acid amplification, the RNA extracted from three of the fragments was split into two aliquots, which were amplified independently. In all cases, the sequences obtained from the two independent amplifications overlapped. These findings indicate that cells within the memory B cell pool, which are already somatically mutated, upon antigenic stimulation can accumulate additional mutations, presumably during the course of giving rise to a further generation of memory B cells.

Discussion

Sequence analysis of H chain V regions of AFC clones generated in vitro has enabled the identification of two distinct response phenotypes of splenic B cells derived both from naive and from immunized mice. In both instances, the majority of antigen-responsive cells generate AFC clones after a single course of antigenic stimulation, a process that was not accompanied by the accumulation of somatic mutations. A minority of the responses of splenic B cells obtained from both naive and immunized mice did not generate AFC after a single course of in vitro antigenic stimulation and, instead, yielded AFC only after a second course of antigenic stimulation (see Figure 2). These responses were accompanied by the accumulation of somatic mutations in the H chain V regions of the resultant AFC clones.

Several investigators have shown that the V regions of primary B cells are not somatically mutated and that the AFC progeny of these cells do not accumulate somatic mutations, at least within the first week after antigenic stimulation (Clarke et al., 1985; Manser et al., 1987; Allen et al., 1987; Malipiero et al., 1987; Siekevitz et al., 1987; Berek et al., 1991; Jacob et al., 1991, Jacob and Kelsoe, 1992). The present study confirms and extends these conclusions by the demonstration that, following a single T_H-dependent stimulation in a culture system competent to facilitate somatic hypermutation, isotype-switched (IgG producing) AFC clones do not accumulate somatic muta-

tions as late as 13 days after stimulation. Although L chain V regions were not analyzed, the likelihood of their having accumulated somatic mutations is low, since the λ chains of anti-NP antibodies accumulate far fewer mutations than H chains and somatic mutations in λ chains have not been found in antibodies lacking H chain mutations (Bothwell, 1984; Allen et al., 1987).

We and others have used disparities in the level of expression of cell surface HSA to separate naive B cells that generate primary AFC clones (HSA^{hi}) from those (HSA^{lo}) that, after a single course of T_H-dependent antigenic stimulation, generate memory B cells rather than AFC (Linton et al., 1989, 1992; Yin and Vitetta, 1992; Wu and Ward, 1993). Earlier studies using SCID mice reconstituted with T_H and various naive B cell subsets demonstrated that cells enriched by virtue of their low expression of HSA were uniquely competent in reconstituting memory and GC responses, and also enabled the generation of hybridomas with somatically mutated immunoglobulin V region genes (Linton et al., 1989, 1992). Unlike suspension cultures, fragment cultures appear to retain sufficient splenic architecture to enable GC formation and the generation of memory B cells. Indeed, recent studies (Linton et al., unpublished data) have demonstrated the acquisition of $I\text{-}E^k$ on B cells derived from fragment cultures reconstituted with HSA^{lo} B cells obtained from B6–Sma58 transgenic mice, which express the transgene-encoded $I\text{-}E^k$ molecule only on GC B cells (van Ewijk et al., 1988). Therefore, it was anticipated that, since fragment cultures enable the generation of memory B cells from stimulated HSA^{lo} B cells, these cultures might also facilitate the in vitro generation of somatic mutations.

The studies presented in this report demonstrate that fragment cultures can support somatic hypermutation and thereby can provide in vitro clonal analysis of the accumulation of somatic mutations during the course of memory B cell generation. In addition to providing an experimental model that will enable the in vitro analysis and manipulation of the mechanism of somatic mutation, these findings reaffirm the conclusion that, prior to immunization, HSA^{lo} memory progenitors represent a nonmutated naive B cell subset distinct from naive AFC precursors. However, although HSA^{lo} memory progenitors and HSA^{hi} AFC precursors coexist within the population of mature B cells of nonimmune mice, the maturational stage at which these distinct functional B cell subsets diverge remains to be resolved. This is particularly the case since recent studies suggest that all B cells express very high levels of HSA as they emerge from the bone marrow and that the population of $HSA^{very\ hi}$ cells has the capacity to reconstitute both AFC and memory B cell responses (Allman et al., 1992).

Although it is clear from these and previous studies (Linton et al., 1989) that naive HSA^{lo} precursors are enriched for cells that give rise to somatically mutated memory B cells, whether there is also a potential role of at least some primary AFC precursors in the generation of memory B cells remains controversial. This possibility has been most recently suggested by Jacob and Kelsoe (1992), who observed that, in some instances, somatically mutated H chain V region genes of cells isolated from splenic GC 10 days after immunization shared HCDR3 with unmutated H chain V region genes of cells from a neighboring AFC focus. Although these findings strongly suggest that both GC and AFC can originate from the same naive precursor cell, their findings do not discriminate between the possibility that the GC originated from the AFC focus or, the alternative possibility, that the unmutated AFC arose from early unmutated GC cells (Berek and Ziegner, 1992; Klinman, 1994). Although somatic mutations were not found in sequences derived from in vitro generated primary AFC clones, the current studies do not directly address the issue of whether precursors of primary AFC clones can also generate somatically mutated memory B cells, since the RNA of the AFC would likely be far more abundant than that of memory cells that might also be present in the same fragment. However, the demonstration (see Figure 3) that antigen-responsive memory AFC precursors with unmutated H chain V region genes had been present within each memory cell clone lends further support to the possibility that cells derived from GC could have given rise to unmutated AFC foci.

Both antibody affinity and the extent of V region somatic mutation are increased, not only during the generation of secondary B cells after initial antigen priming, but also after subsequent courses of antigenic stimulation (Klinman et al., 1966; Berek and Milstein, 1987; Shlomchik et al., 1989). However, it remains highly controversial whether this progressive maturation of antibody responses is due to additional rounds of somatic mutation that could accompany the sequential propagation by memory B cells of further generations of memory B cells or, alternatively, whether this merely represents the further selection of cells with mutations accumulated after the first antigenic stimulation. The latter conclusion is consistent with analyses of previously immunized mice, which suggested that GC arising after subsequent stimulations originate from naive B cells rather than memory B cells (MacLennan and Gray, 1986; Gray, 1993). Since GC are the site of memory B cell generation and somatic hypermutation, these findings would imply that memory B cells neither propagate further generations of memory cells, nor should they acquire further somatic mutations. The conclusion that somatically mutated memory B cells do not accumulate further somatic mutations was also suggested by studies of Siekevitz et al. (1987), wherein AFC derived from somatically mutated memory B cells, which had been restimulated in irradiated adoptive hosts, shared somatic mutations with the donor memory B cells but acquired few additional mutations. These findings are less than compelling, however, since the memory B cells were restimulated using anti-idiotypic antibody rather than antigen. Additionally, the possibility exists that the donor B cells might have been stimulated in sites within the irradiated recipients incapable of supporting somatic hypermutation.

Since the in vitro culture system used for the present studies is fully competent to support somatic hypermutation, the absence of further mutations in AFC clones derived after a single stimulation of somatically mutated memory B cells represents a clear demonstration that the generation of AFC clones from memory B cells is not ac-

companied by somatic hypermutation. The finding that somatic mutations do not accumulate during the generation of AFC clones from either primary or memory B cells is consistent with the conclusion that somatic hypermutation is a process confined to the generation of memory B cells. This finding also suggests that the somatic mutations that were observed in the H chain V regions of AFC clones derived from HSA^{lo} memory progenitors were acquired after the initial in vitro stimulation of these cells, which presumably led to the generation of memory B cells rather than following the second in vitro stimulation, which resulted in AFC formation from these memory cells (see Figure 2).

The paradox created by the fact that, although AFC generated from memory B cells do not accumulate further somatic mutations, memory responses progressively affinity mature and accumulate mutations with subsequent immunizations, is resolved by the unexpected finding that some memory B cells required two courses of stimulation to generate AFC responses and that such AFC had accumulated additional somatic mutations. This finding represents the demonstration that higher order (tertiary, quaternary, etcetera) memory B cells can be generated from previous generations of memory B cells rather than by selection of memory cells generated by the first antigenic priming. It is critical for the validity of this conclusion that the cells that gave rise to memory cells in these experiments were indeed memory B cells. This is clearly the case, since the HSA^{lo} cells were obtained from previously immunized mice, and the frequency of λ-bearing cells that responded only after two courses of stimulation was 10-fold higher than the frequency for comparable populations obtained from naive mice; all AFC clones had shared mutations, indicating that H chain V regions of the HSA^{lo} cells that originated the clones were already somatically mutated; and several of the immune HSA^{lo} progenitors, whose progeny accumulated somatic mutations and responded to a second course of in vitro stimulation, were isolated as either $sIgM^-$ or $sIgD^{lo}$, or both, and, therefore, were already isotype switched.

For all AFC clones that were generated only after two courses of in vitro stimulation, at least one sequence was obtained that had only shared mutations, and thus presumably represented the H chain V region sequence of the memory B cell that originated the clone. The pattern of the superimposed in vitro accumulated mutations in several of these AFC clones was indicative of the sequential accumulation of mutations through several rounds of division. However, the extent to which mutations were accumulated varied widely from clone to clone. Indeed, the H chain V regions of 2 of the 10 analyzed clones had only shared mutations and, therefore, did not appear to have accumulated any mutations after either stimulation or restimulation in fragment cultures. The possibility that memory precursors may ultimately cease accumulating somatic mutations has been suggested by previous investigators (Siekevitz et al., 1987; Shlomchik et al., 1989).

Unlike naive spleen cell populations, wherein memory progenitors and AFC precursors can be separated by virtue of their disparate expression of HSA, memory B cells are enriched in the HSA^{lo} subset whether they behave as AFC precursors or as progenitors of memory cells. Because of the absence of a discriminatory marker, memory B cells that generate higher order memory cells and accumulate somatic mutations versus memory AFC precursors in the same donor cell population could be distinguished only by the criteria of whether AFC clones were generated after one or only after two courses of in vitro stimulation. Therefore, it is possible that whether a given memory B cell generated an AFC clone or higher order memory B cells might have been, at least in part, dependent on its localization within the spleen of the irradiated host from which the fragment cultures were derived. Alternatively, the two distinct response phenotypes observed for memory B cells could indicate that, as is the case for naive B cells, two distinct precursor cell subsets coexist within the memory cell pool, one of which, upon stimulation, can only generate AFC without further somatic mutation, while the other subset generates further generations of memory B cells that continue to accumulate mutations. This latter hypothesis is depicted in Figure 2, wherein the existence of two distinct subsets of both naive and memory B cells is proposed. By this hypothesis, both naive and immune AFC precursors can give rise only to AFC clones, whereas the progeny of both naive and immune memory progenitors accumulate somatic mutations, with the naive memory progenitors giving rise to secondary B cells and immune memory progenitors giving rise to the further generations of memory B cells that propagate memory responses.

Experimental Procedures

Cell Isolation

Spleen cells were isolated from either naive 2- to 4-month-old C.B20 mice (obtained from The Scripps Research Institute breeding colony) or 4-month-old C.B20 mice that had been immunized intraperitoneally and subcutaneously at the base of the tail and at the shoulders 1–2 months previously with 0.1 mg of NP_{10}–Hy in complete Freund's adjuvant. The isolation of HSA^{lo} cells and T_H cells has been described in detail elsewhere (Linton et al., 1989). After red blood cell lysis with Tris-buffered ammonium chloride, B cells were enriched by incubation at 5×10^7 cells/ml with a 1:100 dilution of anti-Thy-1 antibody in ascites and a 1:20 dilution of rabbit complement (Accurate Chemical) for 30 min at 37°C. HSA^{lo} cells were prepared by treating enriched splenic B cells at 4×10^7 cells/ml with a 1:7 dilution of J11d culture supernatant and a 1:20 dilution of rabbit complement for 30 min at 37°C. This treatment was repeated twice, yielding approximately 15% of the initial enriched B cell population. In some instances, subsets of HSA^{lo} B cells derived from immune mice were further enriched using a FACStar Plus cell sorter (Becton Dickinson) for cells that were positive for Ia expression after staining with phycoerythrin–mouse anti-Ia^d (Pharmingen) and were negative for expression of cell surface κ light chains using biotin–rat anti-mouse κ (AMAC, Incorporated) followed by fluorescein isothiocyanate–streptavidin (Biomeda); and/or low to negative for expression of sIgD using biotin–mouse anti-IgD^b (Pharmingen) followed by APC–streptavidin (Biomeda); negative for expression of sIgM using Texas red anti-mouse IgM (Southern Biotech). T_H cells were isolated from the inguinal lymph nodes of BALB/c mice that had been immunized subcutaneously at the base of the tail with 0.1 mg of Hy in complete Freund's adjuvant 5–6 days earlier. $CD8^+$ T cells and B cells were removed by panning on petri dishes coated with goat anti-mouse immunoglobulin (Southern Biotechnology Associates) following the pretreatment of cells with 0.3 μl of ascites containing mouse anti-CD8 per 10^6 cells for 30 min on ice. Less than 2% of the remaining nonadherent cells were either B cells or $CD8^+$ T cells.

Splenic Fragment Cultures

The fragment culture technique was modified as described previously (Linton et al., 1989, 1992) to enable the generation and subsequent stimulation of memory B cells. This was accomplished by the intravenous transfer of 2×10^6 Hy-primed T_H along with limiting numbers of C.B20 cells of various B cell subsets to lethally irradiated (1300 rads) recipients. BALB/c mice that were used as recipients had been immunized intraperitoneally with 0.1 mg Hy in complete Freund's adjuvant and boosted 4 weeks later by an intraperitoneal injection of 0.1 mg of Hy in saline. These mice were irradiated and used as recipients 4–8 weeks after the second injection. Recipients were sacrificed 16–24 hr after cell transfer and 1 mm cubes of splenic fragments prepared, placed individually in microtiter wells, and cultured for 2 or 3 days with NP_{10}–Hy (prepared as previously described; Riley and Klinman, 1985) at a concentration of 10^{-6} M for the NP determinant. Culture fluids were collected 6–8 days later and assessed for anti-NP antibody production by enzyme-linked immunosorbent assay (ELISA) using alkaline phosphatase conjugated to goat anti-mouse IgG^+IgA^+IgM (Kirkegaard and Perry) and the fluorescent substrate, 4-methyl-umbelliferylphosphate (Boehringer Mannheim). Positive cultures were further analyzed by ELISA for the absence of anti-Hy antibody production and for the production of λ-bearing IgG anti-NP antibodies through the use of murine isotype or λ-specific rabbit antibodies and detection with alkaline phosphatase–conjugated goat anti-rabbit immunoglobulin (Kirkegaard and Perry). Cultures that were negative for antibody production after initial stimulation were restimulated for 2 days (days 6–8 for naive HSA^{lo} precursors and days 8–10 for HSA^{lo} precursors from immunized mice) and reassayed for anti-NP antibody production 4–6 days later. Splenic B cells isolated from naive mice were stimulated with antigen in vitro for 3 days.

Fragments were chosen for sequence analysis on the basis that they produced at least 2 ng of λ-bearing IgG anti-NP antibody/day (the amount of antibody produced by a clone of 8 or more AFC) and no Hy-specific antibody. Negative fragments produced no detectable antibody (<0.2 ng/day). Fragment cultures derived from total splenic B cells of naive mice that were positive for the production of anti-NP antibodies 8–10 days after a single course of antigenic stimulation were generally harvested for RNA extraction and sequence analysis 13 days after cultures were initiated. Fragment cultures derived from HSA^{lo} splenic B cells of naive mice that were positive for anti-NP antibody production after a second course of in vitro antigenic stimulation were harvested 13–16 days after cultures were initiated. Cultures derived from HSA^{lo} spleen cells of immune mice that produced anti-NP antibodies after a single course of antigenic stimulation were harvested 8 days after cultures were initiated, whereas those that responded only after two courses of in vitro stimulation were harvested 14–16 days after cultures were initiated.

RNA Isolation

Splenic fragments were removed from culture and teased apart to release the cells from the fibrous fragment matrix. Cells were transferred to a sterile 1.5 ml microcentrifuge tube and incubated at 50°C for 45 min in the presence of 600 μl lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM EDTA [pH 8], 0.2% SDS, and 200 μg/ml proteinase K). Cell lysates were extracted twice with phenol:chloroform:isoamyl alcohol (50:49:1) followed by ethanol precipitation of the RNA in the presence of 0.3 M sodium acetate. RNA pellets were washed in 70% ethanol and resuspended in 10 μl 0.1 mM EDTA (pH 8).

RNA Amplification

Cellular RNA was amplified utilizing the 3SR amplification technique as described previously (Gingeras et al., 1990; Guatelli et al., 1990; Decker and Klinman, 1993; Stillman et al., 1994). In brief, reaction mixtures of 5 μl RNA, 10% DMSO, 15% sorbitol, 40 mM Tris–HCl (pH 8), 30 mM $MgCl_2$, 20 mM KCl, 4 mM spermidine, 10 mM DTT, 6 mM each ribonucleotide, 1 mM each deoxynucleotide, and 0.1 μM of a 5′ oligonucleotide primer 5′-AATTTAATACGACTCACTATAGGGAAAGGCTTCTGGCTACACCTTCA-3′ (the sequence encoding amino acids 22–30 of the V_H186.2 gene segment with a T7 promoter sequence on the 5′ end) and 0.1 μM of a 3′- oligonucleotide primer 5′-AATTTAATACGACTCACTATAGGGATGGAGTTAGTTTGGGCAGCAG-3′ (the sequence complementary to that encoding amino acids 126–135 of the γ constant region gene segment and containing the T7 promoter sequence at the 5′ end) were incubated at 42°C for 1 hr in the presence of 50 U T7 RNA polymerase (Stratagene), 15 U AMV reverse transcriptase (Life Sciences, Incorporated), and 2 U RNAse H (GIBCO BRL). The resultant RNA product (1/25 of the 3SR reaction) was subjected to cDNA synthesis, wherein reaction mixtures (5 mM $mgCl_2$, 50 mM KCl, 10 mM Tris–HCl [pH 8.3], 1 mM each deoxynucleotide, 40 U RNase inhibitor, 25 U AMV reverse transcriptase and 1/25 of the 3SR product) were incubated at 42°C for 15 min, 92°C for 5 min, and 5°C for 5 min, utilizing the oligonucleotide primer 5′-TCCAAAGCTTGGGGCCAGTGGATAGAC-3′ (the complement of the sequence encoding amino acids 121–127 of the γ constant region).

DNA Amplification

cDNA products were amplified by the PCR as previously described (Sakai et al., 1988; Decker and Klinman, 1993), utilizing oligonucleotide primers 5′-TTGATGAATTCGGTGAAGCAGAGGCCTGGACGA-3′ and 5′-TCCAAAGCTTGGGGCCAGTGGATAGAC-3′, representing the sequence encoding amino acids 33–43 of the V_H186.2 gene segment and the complement of the sequence encoding amino acids 121–127 of the γ constant region, respectively. PCR reaction mixtures (50 mM KCl, 10 mM Tris–HCl, [pH 8.3], 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleotide, 2.5 U Taq polymerase [Perkin Elmer], 40 pmol each primer, and cDNA from the reaction described above) were subjected to an amplification program consisting of 30 cycles of incubation at 92°C for 1 min, 45°C for 1 min, and 72°C for 1 min. Amplified products were ethanol precipitated in 0.3 M sodium acetate, resuspended in water, and digested with 40 U EcoRI and 40 U HindIII restriction enzymes (Boehringer Mannheim).

DNA Sequence Analysis

Digested PCR fragments were ligated into a modified version of the $pBSM13^+$ vector (Stratagene) and transformed into Hanahan-competent DH5α Escherichia coli (Hanahan, 1985). DNA was prepared from individual bacterial colonies by standard miniprep procedures (Holmes and Quigley, 1981) and sequenced as described previously (Linton et al., 1989). Sequences of members of a single responding AFC clone were identified by the unique junction between V_H, D, and J_H gene segments. Gene segments incorporated in H chain V gene rearrangements were determined by closest homology to known germline sequences (Allen et al., 1987; Siekevitz et al., 1987; Ichihara et al., 1989; Gu et al., 1991; Kabat et al., 1991) and nucleotide differences from these germline sequences were scored as mutations. A D region was considered mutated if a single base change increased its length by at least 4 nt. Nucleotide differences from germline occurring in every sequence analyzed from a given AFC clone were classified as shared mutations. Differences in nucleotide sequence among members of a clone were scored as in vitro generated somatic mutation events. Because hybrid genes are often generated from PCR amplification of genes in multigene families, all sequences obtained that could have been an artifact created by one to two aberrant hybridization events were excluded from our analysis of somatic hypermutation. While it is possible that an observed sequence could have been generated by more than two internal priming events, we consider this possibility to be unlikely, as hybridization to this extent was not observed in either the analysis of control RNA or nonmutating splenic fragment cultures.

Experimental error inherent in the amplification (3SR and PCR) and bacterial cloning procedures was determined using the RNA from an NP-specific hybridoma (NP5) which uses a λ L chain and a rearrangement of V_H186.2 (Stillman et al., 1994). Two independent 3SR amplification reactions were done in parallel and then each was split between two cDNA extension reactions. The resulting four cDNA reactions were amplified by PCR and sequence analysis detected between one and five errors in the 15,000 nt sequenced for each reaction. Therefore, the average error rate inherent in the techniques used in this system was 0.25 mutations per thousand bases of nucleotide sequence obtained. To rule out the existence of amplification errors peculiar to RNA obtained from fragment culture AFC, in several instances the RNA derived from a single fragment culture was divided into two aliquots, which were then amplified independently (see Figure 1 and Figure 4). In all cases, overlapping nonmutated and mutated sequences were found in the sequences derived from both aliquots.

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